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Female Contact Activates Male-Specific Interneurons that Trigger Stereotypic Courtship Behavior in *Drosophila*

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SUMMARY

We determined the cellular substrate for male courtship behavior by quasinatural and artificial stimulation of brain neurons. Activation of *fruitless* (*fru*)-expressing neurons via stimulation of thermosensitive *dTrpA1* channels induced an entire series of courtship acts in male *Drosophila* placed alone without any courting target. By reducing the number of neurons expressing *dTrpA1* by MARCM, we demonstrated that the initiation of courtship behavior is significantly correlated with the activation of the transmidline P1 interneurons, the descending P2b interneurons, or both, indicating that these interneurons trigger courtship. Using an experimental paradigm in which a tethered male can be stimulated to initiate courtship by touching his foreleg tarsus to a female's abdomen, we found that P1 neurites of tethered males showed a transient Ca^{2+} rise after tarsal stimulation with the female-associated sensory cues. These observations strongly suggest that P1 neurons are the prime components of the neural circuitry that initiates male courtship.

INTRODUCTION

Male courtship in *Drosophila melanogaster* represents a series of highly stereotyped behavioral acts that include orientation toward the target female, tapping the female abdomen with a foreleg, chasing the female, extending and vibrating a unilateral wing to generate courtship songs, licking the female genitalia, and attempting copulation (Hall, 1982; Spieth, 1952). In a successful attempt, the male fly rides on the female's back and copulates (Hall, 1982; Spieth, 1952). The pattern of each motor act and the order in which it is aligned in the courtship series are mostly invariable across individuals and ages and barely change as the fly gains experience (Bastock and Manning, 1955; Hall, 1982). A male fly placed alone does not commence courtship behavior unless activated by some stimuli associated with potential mating partners through a visual, tactile, auditory, or chemical sensory channel (Hall, 1982; Krstic et al., 2009). Therefore, the neural system that generates the courtship ritual in male *Drosophila* has been regarded as a prototype of the innate releasing mechanism that is selectively activated by the social releasers and, once activated, generates a complete series of behavioral acts with minimal sensory feedback (Tinbergen, 1951) via interplay among the neurons in the central nervous system (CNS). Therefore, at least in theory, the courtship neural center for males must operate properly to generate a complete series of behaviors when it is forcibly triggered by direct stimulation of the commanding neural element (Wiersma and Ikeda, 1964). In fact, Clyne and Miesenböck (2008) have reported that decapitated flies display wing vibration similar to that in the singing posture of courting males when a large number of *fruitless* (*fru*)-expressing neurons in the ventral ganglia are directly stimulated by a light-activatable $P2X_2$ channel. Kimura et al. (2008) have demonstrated that ectopic formation of male-specific P1 interneurons in the female brain renders the females more likely to display male-type courtship behavior toward a target female. However, it remains unknown how the brain P1 neurons respond to sociosexual stimuli and activate the neural circuit in the ventral ganglia to produce motor outputs.

In the present study, the direct activation of *fru*-expressing neurons via stimulation of thermosensitive *dTrpA1* channels (Hamada et al., 2008) induced a complete series of courtship acts in male *Drosophila* flies placed alone without any courting target. We used mosaic analysis with a repressible cell marker (MARCM; Lee and Luo, 1999) to reduce the number of neurons expressing *dTrpA1* channels and demonstrated that the initiation of courtship behavior correlates significantly with the activation of the transmidline P1 interneurons, the descending P2b interneurons, or both, indicating that these interneurons trigger courtship. To investigate the neural correlates of courtship behavior, we invented a new experimental paradigm in which male *Drosophila* courtship rituals can be initiated and observed by placing a tethered male on an air-jet-supported Styrofoam ball, causing him to walk stationarily, and then touching his foreleg tarsus with a female abdomen. Ca^{2+} imaging of *fru*-expressing neuron activities in such tethered males demonstrated that P1 neurites in the lateral protocerebrum immediately and transiently increase the Ca^{2+} concentration in response to the tarsal stimulation with the female-associated sensory cues. These observations strongly support the hypothesis that P1 neurons are the prime components of the neural mechanism that initiates courtship in *Drosophila* males.

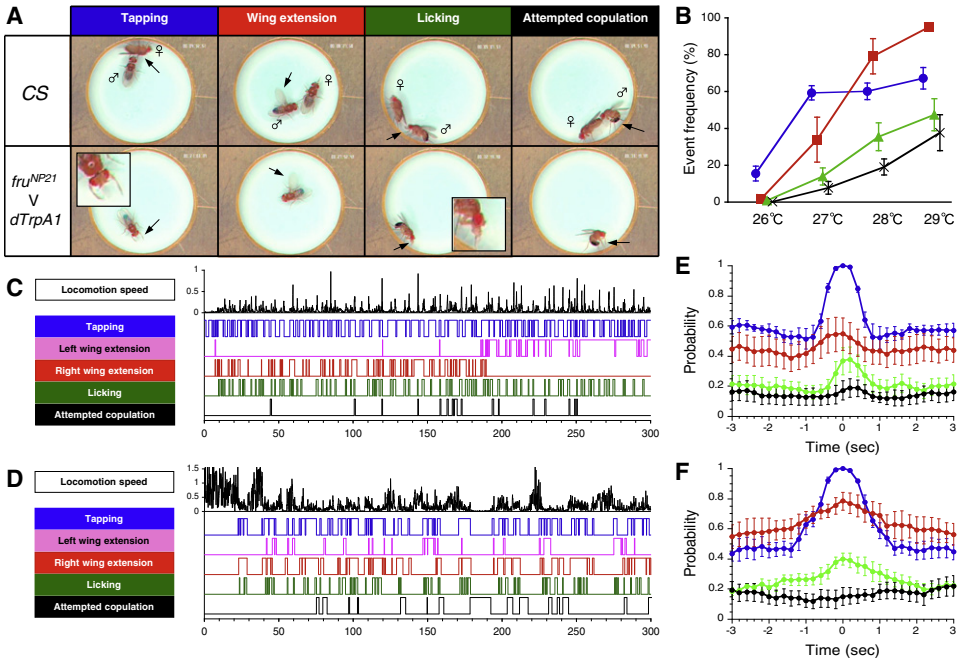

Figure 1. Induction of Courtship Behavior in a Male Fly Placed Alone by Activation of *fru*-Expressing Neurons via *dTrpA1* with a Temperature Increase

(A) Courtship acts (arrows) induced by *dTrpA1*-mediated activation compared with those observed in a wild-type (CS) male courting a female.

(B) The temperature dependence of the frequency (mean ± SEM; n = 5) of tapping (blue circles), wing extension (red squares), licking (green triangles), and attempted copulation (black crosses) in males stimulated via *dTrpA1* during a 5 min observation period.

(C and D) Ethogram showing the temporal organization of different courtship acts in a male fly expressing *dTrpA1* under the control of fru^{NP21} without a target female at 27°C (C) and that in a male fly courting an immobilized female (D). Top traces show the locomotion speeds for every consecutive 100 ms. Each block contains 5 min records of events. Each bar (upward deflection) corresponds to the occurrence of a single act. Time elapsed is indicated at the bottom (in seconds).

(E) and (F) Perievent histogram analyses of *dTrpA1*- and female-induced courtship (mean ± SEM; n = 5). The behavior was decomposed into four elements: tapping (blue), wing extension/vibration (red), licking (green), and attempted copulation (black).

RESULTS

Male Courtship Is Initiated by Direct Activation of *fru*-Expressing Neurons in the Absence of a Courtship Target

The male flies with the transgenic *dTrpA1* expression in *fru*-expressing neurons displayed most components of the courtship sequence, i.e., tapping, unilateral wing extension and vibration, licking, and attempted copulation, even when they were placed singly in a mating chamber ("single male assays"), provided that the ambient temperature was increased from 22°C to 30°C (Figures 1A; Figures S1A and S1B and Movie S1, available online). *dTrpA1* had robust power for inducing courtship behavior, i.e., 100% of tested flies exhibited most of the elementary courtship acts in response to temperature increases (Figure 1B). Tapping occurred prior to other acts at lower temperature ranges: it was typically elicited within 2 min (113.7 ± 44.3 s, mean ± SEM, n = 15) upon a temperature increase from 25°C to 26°C (Figure 1B). Other acts were activated at temperatures higher than 26°C (Figure 1B). Under our experimental conditions, wing extension, licking, and attempted copulation commenced 14.3 ± 56.3 s, 31.5 ± 31.0 s, and 142.5 ± 59.2 s (mean ± SEM, n = 15) after a temperature increase from 26°C to 27°C, respectively. As the temperature increased, the frequency of occurrence of each behavioral act increased (Figure 1B). When the temperature was decreased back to 22°C, the male flies stopped courting.

Each unitary behavioral act induced in singly placed males by the *dtrpA1*-mediated activation of *fru*-expressing neurons was indistinguishable from the corresponding behavioral act exhibited by normal males courting a female (Figure 1A). The temporal patterns of the occurrence of tapping, wing extension and vibration, licking, and attempted copulation were recorded separately as digital data, aligned with locomotor activity records, and compared between a wild-type male paired with an immobilized female (Figure 1D) and a male in which *fru*-expressing neurons were artificially activated with *dTrpA1* (Figure 1C). To characterize the temporal relationship among elementary acts, we constructed peri-event histogram plots (Figures 1E and 1F and Figure S1). Regardless of whether the male flies were activated via *dTrpA1* (Figure 1E) or stimulated with an immobilized female (Figure 1F), they typically exhibited all four courtship elements at nearly the same time; the mean peak time at which the frequency of occurrence of events reached the peak was calculated for tapping (defined as time 0), wing extension, licking, and attempted copulation, and no

statistical difference was detected in these values among the four courtship acts ($p > 0.05$, Kruskal-Wallis ANOVA).

A previous study has shown that female flies can exhibit male-type courtship behavior if *fru*-expressing neurons are directly activated by optical stimulation of $P2X_2$ channels targeted to these cells (Clyne and Miesenböck, 2008). In keeping with this, females displayed male-type courtship behavior when their *fru*-expressing neurons were directly activated by *dTrpA1*, although, as in $P2X_2$-mediated activation, stronger stimuli (i.e., approximately 2°C higher temperature than in males) were required for the successful elicitation of behavior (Figures S1E and S1F).

P1 and P2b Neurons May Be Involved in Command Functions in Male Courtship

To determine which subset of *fru*-expressing neurons plays a role in triggering the motor program for male courtship, we performed behavior assays with male flies in which transgenic *dTrpA1* expression was restricted to a limited number of *fru*-expressing neurons by means of MARCM (Lee and Luo, 1999). To improve the efficiency of the analysis, we employed a prescreening for the mosaic males that responded to temperature increases with changes in wing motion to increase the number of flies that could display courtship behavior if *dTrpA1* was activated. Among the 474 mosaic males subjected to the prescreening, 61 flies reacted to temperature increases with wing motion (prescreening-positive flies, herein abbreviated as prepositive flies). Remaining 413 mosaic males did not respond to temperature increases with wing motion (prenegative flies). All 61 pre-positive flies and the same number of pre-negative flies were then subjected to a detailed examination of behavior under a temperature increase from 22°C to 35°C. We conducted an in-depth analysis of video-recorded behavior for these 122 flies, yielding the flies that displayed, upon a temperature increase, unilateral wing extension and/or tapping, two of the hallmarks of male courtship behavior (Movie S2). As a few brains from these flies were lost before the anatomical examination, we ultimately recovered 102 mosaic flies that successfully yielded both behavioral and neuroanatomical data. We obtained 46 mosaic males (courters) that exhibited unilateral wing extension characteristic of courtship behavior and 56 individuals that did not display unilateral wing extension (noncourters). In the detailed behavior examination, we also scored the flies for tapping instead of unilateral wing extension; we obtained 60 courters that showed tapping and 42 noncourters that did not show tapping. We then compared the proportion of brains with *dTrpA1*-positive cells in each neuron cluster between the courters and noncourters. We anticipated that, if a given neuronal cluster plays a key role in male courtship behavior, then the courter group would have a significantly greater number of flies with *dTrpA1* expression in that neuronal cluster than the noncourter group. Among the 27 *fru*-expressing neuronal clusters scored in this study, only five (unilateral wing extension; left-side graph) or three (tapping; right-side graph) clusters showed significantly different frequencies of *dTrpA1* expression between the groups (Figure 2A). Intriguingly, two clusters, P1 and P2b, were *dTrpA1*-positive at significantly higher rates in courters than noncourters in both the unilateral wing extension and tapping trials (Figure 2A). When unilateral wing extension was used for the analysis, P1 and P2b were positive for *dTrpA1* in 54.3% (25/46) and 60.9% (28/46) of courters, respectively, versus 5.4% (3/56) and 8.9% (5/56) of noncourters, respectively. Mosaic flies that bore GFP-positive P1 or P2b made up 87.0% (40/46) of the courter group, and 28.3% (13/46) of courters were doubly GFP-positive for P1 and P2b. Most of the GFP-positive P1 or P2b clones were formed unilaterally in the brains of the courters, except in the case of three courters that carried bilateral P1 clones and three courters that carried bilateral P2b clones. This indicates that both P1 and P2b are domineering in inducing courtship behavior. Importantly, the mosaic flies with GFP-positive P1 or P2b typically started their courtship attempts with tapping and then displayed wing extension and vibration (Figure 2B). With our temperature-shift regime, the levels of courtship activities elicited in male flies with P1 clones expressing *dTrpA1* were generally high, yielding a mean wing extension index (the percentage of time a male exhibits wing extension in a 5 min observation period) of 45.5 ± 6.1 (mean ± SEM, n = 25), a value comparable to that obtained with wild-type male flies courting an immobilized female (42.4 ± 4.9, n = 10).

In another set of MARCM analyses, *shibire^{ts}* (*shi^{ts}*) was expressed in clones of brain neurons so that their synaptic activities could be conditionally blocked by an increase in temperature (Kitamoto, 2001). In this series of experiments, single males with MARCM clones were paired with a female first at a restrictive temperature, 30°C, at which the mating behavior was recorded. The ambient temperature was then decreased to 20°C and the male's behavior was again recorded. Finally, the assayed male flies were sacrificed for histological examination of the brain to identify the GFP-positive and thus *shi^{ts}*-positive neurons. The flies were classified into three groups, i.e., males with GFP-positive P1s bilaterally (bilateral P1), those with GFP-positive P1s unilaterally (unilateral P1), and those without GFP-positive P1s (no P1), and the average courtship index (CI) values among these three groups were compared. At the restrictive temperature of 30°C, the CI for the bilateral P1 group was significantly lower (11.5 ± 7.3, mean ± SEM) than those for the unilateral P1 and no P1 groups (70.3 ± 6.9 and 74.8 ± 5.9, respectively; Figure 2C). At the permissive temperature of 20°C, the CI for the bilateral P1 group greatly increased (47.2 ± 3.9), although this value was significantly lower than the CI values for the unilateral P1 and no P1 groups at 20°C (62.4 ± 6.8 and 66.8 ± 3.8, respectively; Figure 2C), presumably because a preceding exposure to the restrictive temperature had sustained inhibitory effects on these flies. On the other hand, bilateral inhibition of P2b rather moderately, yet significantly, reduced male courtship activities (Figure 2C).

P1 is a male-specific neuronal cluster, the artificial production of which in the female brain causes the female to display male-typical courtship behavior (Kimura et al., 2008). Nothing is known about the functions of P2b neurons. However, P2b neurons extend long axons that descend to the ventral ganglia, implying that they relay trigger signals for motor pattern generators (Figures 2E and 2F). In fact, P2b neurons have arborizations in the subesophageal ganglion as well as in pro-, meso-, and metathoracic segments of the ventral ganglia (arrows in Figures 2E and 2F), the regions implicated as the sites of motor pattern

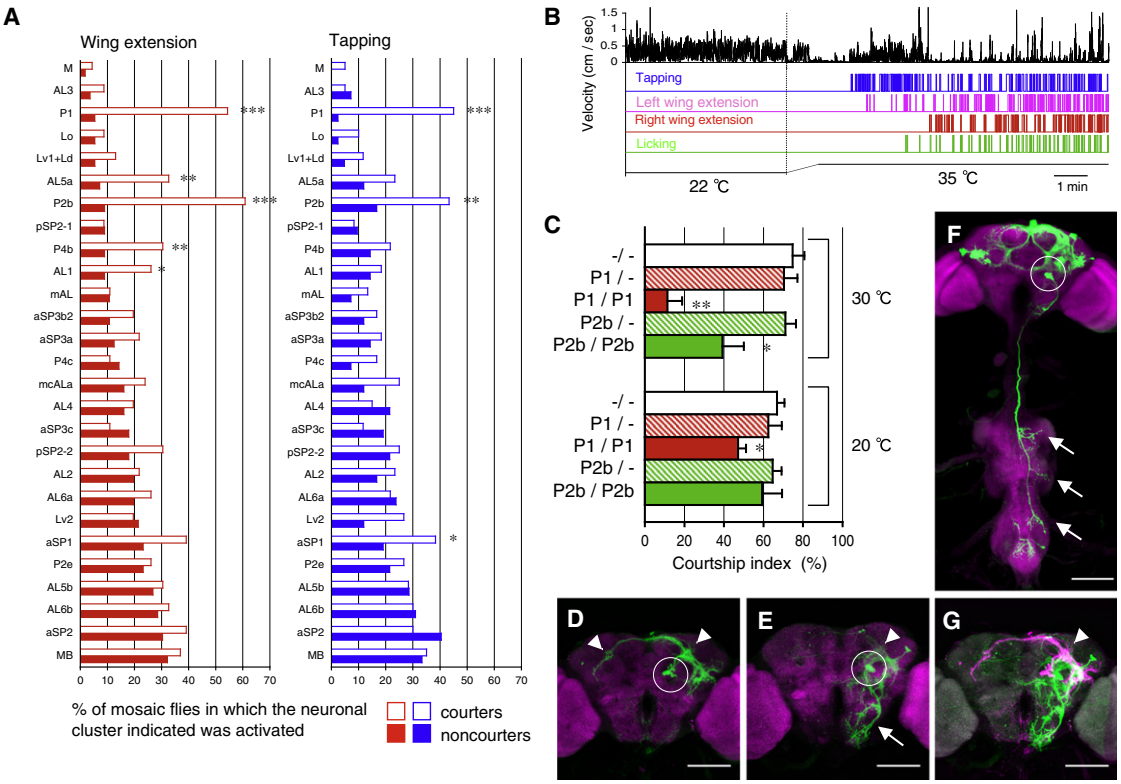

Figure 2. Identification of Neurons, the Activation of Which Is Correlated with the Generation of Courtship

(A) The proportions of mosaic flies in which the indicated cluster was positive for *dTrpA1* were compared between the groups showing courtship (open bars) and those not showing courtship (filled bars) for wing extension and tapping (***p < 0.001, **p < 0.01, *p < 0.05 by Fisher's exact probability test).

(B) Ethogram of a male fly with a *dTrpA1*-expressing P1 clone.

(C) Effects of shi^{ts} on courtship. The CI (mean ± SEM) was compared between five fly groups: −/− (n = 10), no shi^{ts}-expressing clones; P1/- (n = 10), shi^{ts} in unilateral P1; P1/P1 (n = 4), shi^{ts} in bilateral P1; P2b/- (n = 10), shi^{ts} in unilateral P2b; and P2b/P2b (n = 5), shi^{ts} in bilateral P2b. **p < 0.01, *p < 0.05 by the Mann-Whitney U test.

(D and E) P1 (D) and P2b (E) in the flies showing courtship upon a temperature increase. The brain was doubly stained with anti-GFP (green) and Mab nc82 (magenta). Somata (circles), presumptive output sites (arrows), and arborizations in overlapping regions (arrowheads) are indicated.

(F) The entire structure of P2b.

(G) Close apposition of P1 (magenta) and P2b (green) arborizations (arrowheads). The image of a P2b mosaic brain was reformatted on that of a P1 mosaic brain. The neuropil stained with nc82 is shown in gray.

Scale bars represent 100 μm.

generation (von Schilcher and Hall, 1979). It is of interest that the P1 arbors are superimposed on the P2b arbors in the lateral protocerebrum (arrowheads in Figures 2D, 2E, and 2G, and Movie S3). Together, the results of the present study using direct neuronal excitation and those of the previous study using focal neural masculinization (Kimura et al., 2008) highlight the importance of the P1 cluster in initiating male courtship behavior.

Chemical Stimuli Release Courtship Behavior in a Tethered Male

To examine whether or not P1 neurons are activated in a context in which males normally initiate courtship, we developed a system that allows recording of neural activities from a male under conditions that stimulate courtship behavior. In this system, a male fly is tethered with a metal wire on his dorsal thorax while walking stationarily on a Styrofoam ball supported by an air jet (Figures 3A–3C, Figure S2A, and Movie S4). The trajectories of locomotion are recorded by an automated tracking system (Figure 3D and Figure S2). When the foreleg tarsus of the resting tethered male is touched by the abdomen of a virgin female, he typically responds by orienting his body axis toward the side of the stimulated foreleg and then ceasing his movement if the target virgin female is immobile. If the stimulus virgin female is moved to the left and to the right in front of the male after the touch on his foreleg under white light conditions, he starts running to follow the female, producing a zigzag trajectory that is recorded on the automated tracking system (Figures 3E and 3F, Figure S2B, and Movie S4). In contrast, a male abdomen presented in the same way does not induce courtship responses in tethered males (Figure 3I). The tethered male activated by a virgin female extends and vibrates a wing on the side where the virgin female abdomen is presented under white light illumination. When the stimulus is moved from

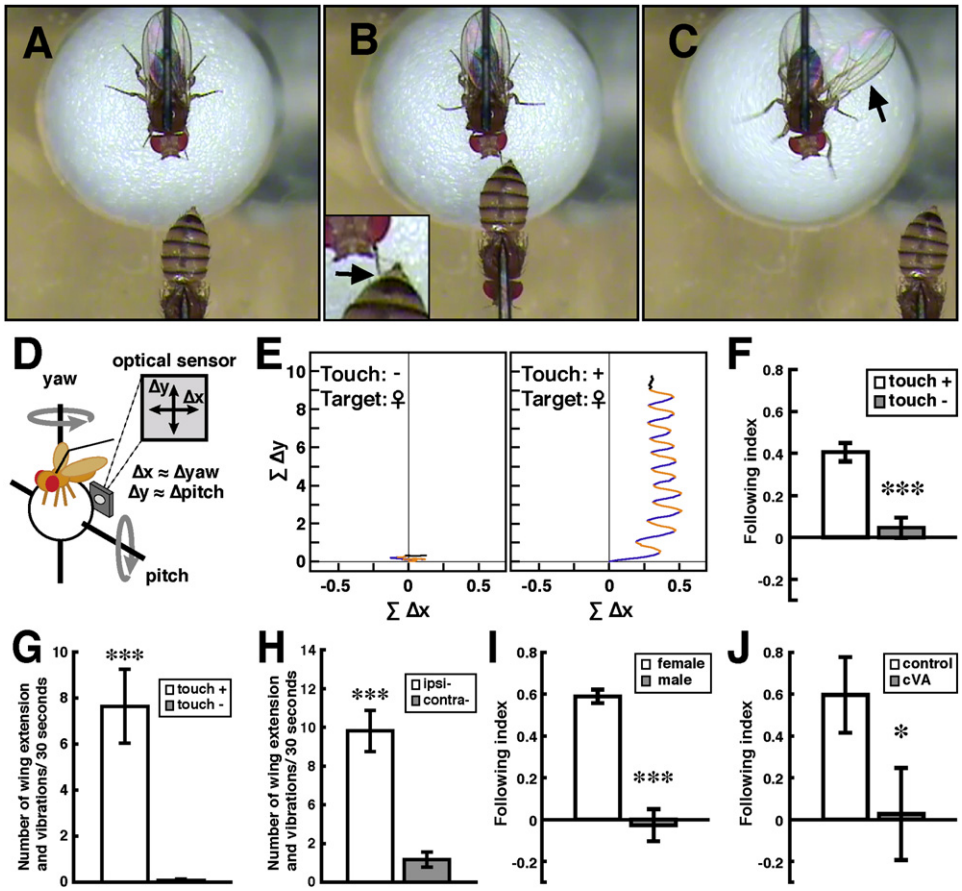

Figure 3. Quantification of Courtship

(A) The female is presented in front of the male's head without inducing a discernible reaction in the male.

(B) The female is touched once to the foreleg tarsus of the male (arrow).

(C) After the contact, the male follows the female and extends a wing on the side to which the female is presented (arrow).

(D) The recording system for the locomotor trajectory.

(E) Plots of the lateral (yaw, $\Sigma\Delta x$) and forward (pitch, $\Sigma\Delta y$) movements with ("touch +") or without ("touch –") a female touch. Trajectories were shown in blue (right) or yellow (left) depending on the side of target presentation.

(F and G) Quantification of following (F) and wing vibration (G) in the male with ("touch +"; n = 26) or without ("touch –"; n = 42) a preceding tarsal contact (see Experimental Procedures for definition of the following index).

(H) Correlations between the side of virgin female presentation and the side on which the male vibrates a wing (n = 26).

(I) The effectiveness of the virgin female abdomen and the male abdomen to induce following responses (n = 12 for females and 8 for males).

(J) Effect of cVA on following responses (n = 11). The means ± SEM are shown for all data. Statistics used are the Mann-Whitney U test for (F), (I), and (J) and Wilcoxon signed rank test for (G) and (H) (***$p < 0.001$, *$p < 0.05$).

one side to the other, the tethered male changes the extended and vibrated wing accordingly (Figures 3G and 3H). This is what has been reported for normal courtship (Hall, 1982). Interestingly, the tethered male does not persistently follow the moving female unless a touch on his foreleg by the virgin female abdomen precedes the presentation of the female (Figures 3E–3G).

Mating males synthesize *cis*-vaccenyl acetate (cVA), which is transferred, during copulation, to the female and acts to reduce her sexual attractiveness (Everaerts et al., 2010; Jallon et al., 1981). The tethered males were given a touch on their forelegs with a stimulus virgin female abdomen on which either 200 ng of cVA dissolved in 50% ethanol (EtOH) or 50% EtOH alone was deposited, and then another virgin female abdomen that had not been treated with either 50% EtOH or cVA solution was presented as a visual target. When a virgin female abdomen coated with cVA was used to touch the tethered male foreleg, the subsequent following response to another virgin female without cVA deposits was significantly suppressed (Figure 3J). On the other hand, presentation of a moving glass rod after contact with a glass rod coated with cuticular extract does not elicit a sustained following response in the male. These observations show that, under the current experimental conditions, the sensory information acquired by contact with the target is sufficient for courtship initiation in a male, while additional sensory cues are required for continued courting.

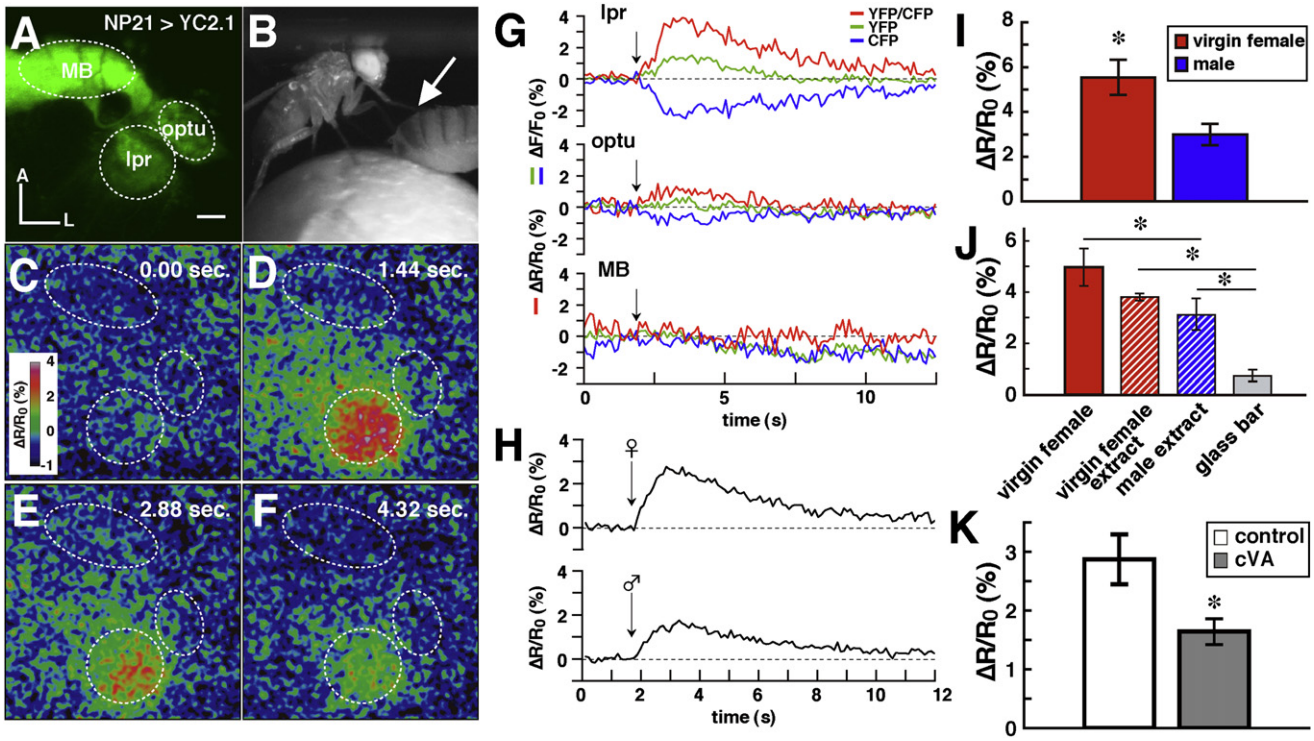

Figure 4. Neuronal Correlates of Courtship

(A) YC2.1-derived YFP fluorescence detected in the mushroom body (MB), lateral protocerebrum (lpr), and optic tubercle (optu). A projected image of nine serial confocal sections with 1.2 μm interval is shown, dorsal view. A, anterior; L, lateral. The scale bar represent 20 μm.

(B) The male touches his foreleg to the virgin female (arrow).

(C–F) Pseudocolor images of ratio changes in *fru* neurons in response to a single female touch. Time after the stimulus is shown on the top right.

(G) The time courses of changes in the dual-wavelength fluorescence emission from YC2.1 before and after the stimulation (arrow) by a female touch observed at lpr, optu, and MB (nonaveraged single responses).

(H) Single Ca^{2+} responses induced by a female touch (upper panel) or a male touch (lower panel).

(I) Quantification of lpr Ca^{2+} responses (n = 10) induced by either a virgin female or a male.

(J) Average Ca^{2+} activity changes in the lpr induced by a touch with a virgin female, a glass rod coated with virgin female extract, a glass rod coated with male extract, or a plain glass rod in a single male (n = 6). See also Figure S3.

(K) Effect of cVA on lpr Ca^{2+} responses (n = 7). The mean ± SEM are shown. The Mann-Whitney U test was used for statistics (*p < 0.05). The fly genotype was *w/Y; UAS-Yellow Cameleon 2.1; fru^{NP21}/TM3, Ser*.

Neuronal Correlates of Courtship Behavior Recorded from a Tethered Male

YFP and CFP fluorescence emitted from Yellow cameleon (YC2.1)-expressing *fru*-neurons was detected through the opening of the head cuticle (Figure 4A). During the monitoring of fluorescence from the lateral protocerebrum (lpr), to which P1 neurons extend neurites, and its surrounding area, the foreleg tarsus of the tethered male was brought into contact with a stimulus, either the female abdomen or a glass rod with or without fly cuticular extract (Figure 4B and Movie S5). When a tethered male tarsus was stimulated with a virgin female abdomen, the Ca^{2+} concentration rose rapidly in the *fru*-expressing lpr neurons, whereas few or no changes in Ca^{2+} signals were observed in the optic tubercle or mushroom body (Figures 4C–4G and Movie S5). Furthermore, the Ca^{2+} rise recorded from the lpr *fru*-expressing neurons was twice as large when stimulated by a virgin female abdomen than when stimulated by a male abdomen (Figures 4H and 4I).

The *fru*-expressing lpr neurons responded similarly, with a rapid Ca^{2+} rise, to a tarsal touch by a glass rod coated with fly cuticular extract (Figure 4J and Figure S3). A touch with a glass rod not coated with fly cuticular extract did not elicit a significant response, demonstrating that it was the cuticular components that provoked the Ca^{2+} rise in these *fru*-expressing neurons and that mechanical contact alone was insufficient to excite them.

In this experiment, male extract was equally effective as virgin female extract in inducing a Ca^{2+} rise in *fru*-expressing lpr neurons (Figure 4J), although the male abdomen elicited a Ca^{2+} response that was about half the intensity of that elicited by the virgin female abdomen (Figures 4H and 4I).

It is known that mated females elicit courtship in males less than virgin females do (Siegel and Hall, 1979), partly because some compounds including cVA transferred from a male to the female during copulation exert inhibitory effects on male courtship (Everaerts et al., 2010; Jallon et al., 1981; Scott et al.,

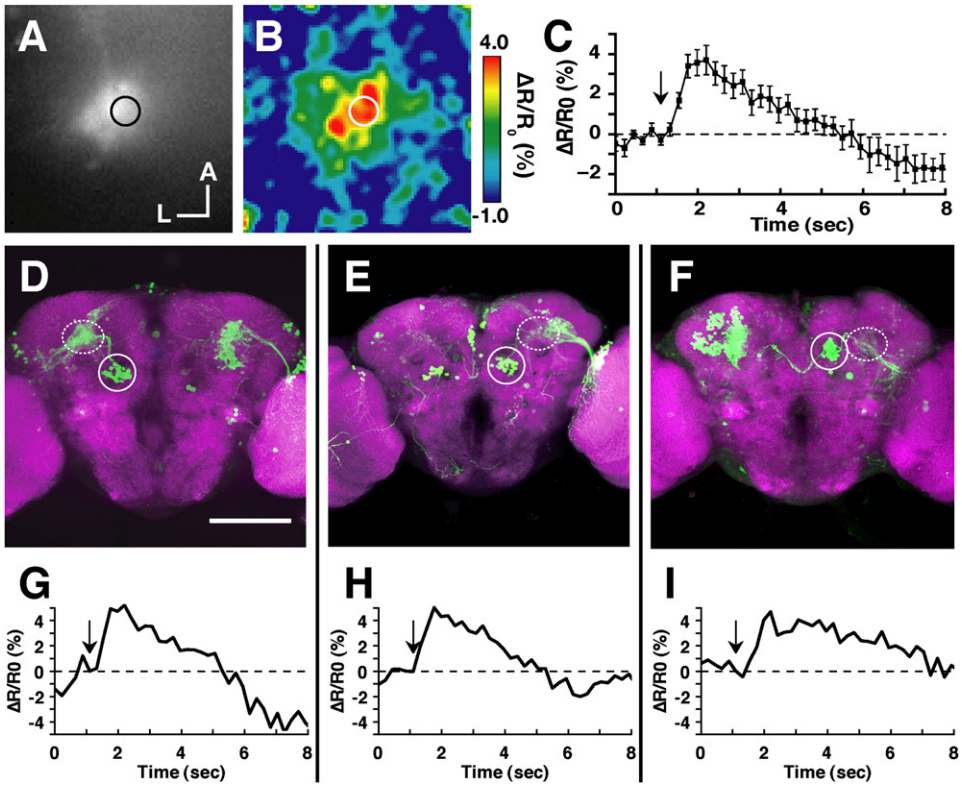

Figure 5. Optical recordings of Ca^{2+} responses in the MARCM clone neurons

(A) A raw YFP fluorescent image of the MARCM clone used for recording. The region of interest (ROI) is indicated by a black circle. A, anterior; L, lateral.

(B) A color-coded response of the MARCM clone 2.02 s after a tarsal contact with a virgin female abdomen. The ROI is indicated by a white circle.

(C) The time course of Ca^{2+} activity changes before and after the stimulation (arrow) of the tethered male foreleg with the virgin female abdomen. Each point represents the mean ± SEM of records from six P1 clusters, each from a different fly. Of these six P1 clusters from which activities were recorded, three are shown in (D)–(F).

(D–F) The brains of the flies from which the records in (C) were obtained, stained for YC2.1 by using an anti-GFP antibody to label the MARCM clones, indicate that the P1 neurons were labeled (circles). The dotted ellipsoid shows the approximate brain region in which the recording was made. Scale bar represents 100 μm. Circles indicate cell bodies of P1 neurons.

(G –I) Ca^{2+} responses obtained from P1 clusters in the brains shown in (D)–(F), respectively. The males were stimulated at the time indicated by arrows.

1988; Yamamoto et al., 1997; Yew et al., 2009). cVA has been shown to abrogate the excitatory action of a female-specific pheromone, 7,11-heptacosadiene (Billeter et al., 2009). As shown in Figure 4K, the Ca^{2+} responses of *fru*-expressing lpr neurons induced by a touch with the virgin female abdomen were significantly attenuated by cVA. This result is in concert with the observation that cVA deposits on the virgin female abdomen suppress the tethered male response to follow a female target (Figure 3J).

To further clarify the role of *fru*-expressing neurons in regulating male courtship, we carried out Ca^{2+} activity imaging of a single neuronal clone expressing YC2.1, as produced by MARCM (Figure 5). We chose male flies whose brains emit YFP fluorescence from the lateral protocerebrum. Among 169 MARCM flies prepared for Ca^{2+} imaging under tethered conditions, 30 had detectable YFP signals in the lateral protocerebrum and were then subjected to Ca^{2+} activity measurements. After Ca^{2+} imaging, the brains were examined for anti-GFP antibody reactivity to identify the recorded cells based on the fact that only the YC2.1-expressing cells were GFP-positive. P1 neurons thus identified in a tethered male showed a rapid rise in the Ca^{2+} concentration in response to the female abdomen in practically the same manner as observed in non-MARCM fly preparations (Figure 5). This result unambiguously shows that the male-specific P1 neurons are immediately activated by female-associated stimuli that were shown to trigger courtship behavior in male flies (Figures 3A–3C and 3E–3G).

DISCUSSION

In this study, we demonstrated that core portions of male courtship behavior are produced even when the male fly is placed alone without a courtship target, if many of the *fru*-expressing neurons are artificially activated by *dTrpA1* simultaneously. By reducing the number of *dTrpA1*-expressing cells by MARCM, two *fru*-expressing clusters, P1 and P2b, were shown to trigger male courtship behavior. The P1 cluster is composed of male-specific interneurons with transmidline neurites (Kimura et al., 2008). P2b represents descending interneurons. Males displayed courtship even under tethered conditions, allowing

Ca^{2+} imaging of single-neuron activities in response to courtship-inducing stimuli. The female abdomen and fly extracts induced rapid Ca^{2+} rises in P1, further supporting the hypothesis that P1 triggers male courtship.

The earliest mosaic analysis of the elements of courtship behavior used gynandromorphs carrying body surface markers and mapped the foci for courtship in the brain (Hotta and Benzer, 1976). Subsequent studies employed gynandromorphs with internal markers (Hall, 1979; von Schilcher and Hall, 1979) or mosaicisms produced by the Gal4-UAS system (Ferveur and Greenspan, 1998; Broughton et al., 2004), narrowing the male courtship foci down to specific regions in the CNS. Intriguingly, Hall (1979) found that the posterior brain site SP3 must be composed of male tissue on at least one side of the brain, in order for a gynandromorphic fly to display male-type courtship behavior. The P1 cluster is located precisely in this region. The fact that, in the present study, P1 neurons responded immediately and transiently to female-associated chemosensory stimuli is consistent with the hypothesis that multiple sensory inputs related to potential targets of courtship converge at P1 neurons, which in turn fire to initiate the neural program for courtship—i.e., P1 operates as the primary switch of courtship behavior. The transmidline neurite of P1 neurons may mediate bilateral coordination of neural activity for courtship.

Sensory pathways converging at P1 neurons have been poorly defined, however. Bray and Amrein (2003) claimed that female cuticular hydrocarbons with aphrodisiac effects on males are perceived by tarsal sensory cells expressing *Gr68a*. However, the majority of *Gr68a*-expressing neurons were later shown to be mechanosensory neurons projecting to the antennal mechanosensory and motor center (AMMC) in the brain (Ejima and Griffith, 2008; Koganezawa et al., 2010), a site remote from the P1 dendritic field (Kamikouchi et al., 2006). On the other hand, *Gr32a*-expressing sensory neurons in the foreleg tarsus have been demonstrated to transduce contact pheromone information that inhibits male-to-male courtship (Miyamoto and Amrein, 2008) and tunes the posture of the male in courting a female (Koganezawa et al., 2010), depending on certain conditions and contexts. The *Gr32a*-expressing sensory neurons terminate in the subesophageal ganglion, where they seem to connect with a sexually dimorphic population of *fru*-expressing neurons, mAL neurons, in male but not female flies (Koganezawa et al., 2010). Interestingly, mAL neurons extend neurites to the lateral protocerebrum (Kimura et al., 2005), where P1 dendrites ramify extensively (Kimura et al., 2008). It remains to be examined whether mAL neurons connect with P1 neurons directly or indirectly.

Apart from contact chemoreception, olfaction is another sensory modality crucially involved in the regulation of sexual behavior (Krstic et al., 2009; Yamamoto et al., 1997). The best-studied olfactory pheromone in *Drosophila* males is cVA (Jallon et al., 1981), which inhibits male courtship (Jallon et al., 1981) and facilitates female sexual receptivity (Ronderos and Smith, 2010). cVA also functions as a reinforcer in conditional, learned suppression of male courtship (Ejima et al., 2007). Two olfactory receptors, *Or65a* and *Or67d*, respond to cVA (van der Goes van Naters and Carlson, 2007). Learned suppression of male courtship involves *Or65a*, whereas the innate aspects of courtship involve *Or67d* (Ejima et al., 2007; Ronderos and Smith, 2010; Stockinger et al., 2005). *Or67d*- and *fru*-expressing sensory neurons in antennal trichoid sensilla innervate DA1 (Stockinger et al., 2005; Kurtovic et al., 2007), one of a few sexually dimorphic glomeruli of the antennal lobe (Kondoh et al., 2003), where they synapse onto a defined set of projection neurons. These postsynaptic projection neurons terminate in the pheromone-responsive region of the lateral horn (Jefferis et al., 2007), with axon arbors whose pattern is sexually dimorphic and *fru* dependent (Datta et al., 2008). It is conceivable that P1 neurons receive cVA-associated inputs from this pheromone-specific olfactory pathway, in view of the fact that P1 is sensitive to cVA (Figure 4K) and in light of the strong anatomical coupling between the lateral horn and neighboring lpr dendritic fields (Strausfeld, 1976; Cachero et al., 2010; Yu et al., 2010).

The trigger signal initiated in P1 is probably sent to the motor centers in the ventral ganglia, presumably as relayed by descending interneurons, and P2b cells are promising candidates for this role. In the present study, we showed that 86% of courter mosaic males had *dTrpA1* expression in either P1 or P2b, or both, indicating that these two groups of neurons together constitute the primary circuitry for initiating male courtship behavior. However, our observation that some of the courtship motions can be generated at a low frequency even without the involvement of P1 and P2b (Figures 2A and 2C) indicate the presence of additional neural elements with the ability to initiate these behavioral acts.

Highly coordinated swimmeret movements across abdominal segments of crayfish can be induced by unpatterned repetitive stimulation of transsegmental nerve fibers called command fibers (Wiersma and Ikeda, 1964). Cricket singing is known to be controlled by similar descending command neurons, the frequency of tonic discharges of which determines the type of songs to be produced in the motor circuits in the thoracic ganglia (Bentley, 1977; Huber, 1978). It remains to be determined whether or not P2b neurons share such command fiber properties. A recent finding (Clyne and Miesenböck, 2008) that the wing extension and vibration characteristic of courtship can be induced by direct stimulation of *fru*-expressing neurons in decapitated flies also supports the localization of the relevant motor center in the ventral ganglia (Rideout et al., 2007).

An important difference between our results and those reported by Clyne and Miesenböck (2008) is in the proportion of flies that displayed male courtship behavior in response to forced activation of *fru*-expressing neurons: 100% in the present study (Figures 1B and Figure S1E) compared to 46% in the previous work (Clyne and Miesenböck, 2008). This might indicate that a brain-derived descending command is required for sustained male courtship. Alternatively, the observed difference in the proportion of responding flies might have a technical origin. For example, in activating *fru*-expressing neurons, Clyne and Miesenböck (2008) utilized caged ATP, which could readily disperse and become ineffective at stimulating the targets. In contrast, when *dTrpA1* is used to activate neurons, excitation would persist as long as the fly is maintained at temperatures that are effective to open *dTrpA1* channels. Thus the approach using *dTrpA1* to activate target neurons is better suited for quantitative analysis of behavior, particularly when assays need to be replicated with a single fly.

In this study, we identified a few neuronal clusters that form the core portion of the neuronal circuitry for male courtship. However, many more intervening neurons must be identified for a complete understanding of the neural circuitry underlying courtship behavior. Electrophysiological and electron-microscopic analyses will be needed to identify the synaptic connections among these neurons. Another group has recently reported success in patch clamp recordings from a visual interneuron in tethered flying *Drosophila* (Maimon et al., 2010). The integration of these two techniques will help to clarify the sophisticated neurogenetics of complex behavior, and thereby facilitate the emergence of a novel conceptual framework for the study of instinct.

EXPERIMENTAL PROCEDURES

Fly Strains and Induction of Neuronal Clones

Flies were reared on cornmeal-yeast medium under a 12:12 light:dark cycle at 25°C, except for those carrying *fru*^{NP21} and *UAS-dTrpA1*, which were reared at 19°C. *UAS-dTrpA1* and *UAS-shi*^{ts} were gifts from P. Garrity and T. Kitamoto, respectively. Other fly stocks were obtained from the Bloomington Stock Center and the Kyoto Stock Center. Somatic clones were produced by using the MARCM method as described previously (Lee and Luo, 1999). The genotype of the fly used for single male assays shown in Figure 1 was *w; UAS-dTrpA1/+; fru*^{NP21}*/+*. The genotypes of flies used for MARCM were *y hs-flp; FRTG13 tub-Gal80/FRTG13 UAS-mCD8::GFP; fru*^{NP21}*/UAS-dTrpA1* (in the analysis of neural clusters involved in male courtship), *y hs-flp; FRTG13 tub-Gal80/FRTG13 UAS-mCD8::GFP; fru*^{NP21}*/UAS-shi*^{ts} (in the analysis of neural inactivation), or *y hs-flp; FRTG13 tub-Gal80/FRTG13; fru*^{NP21} *UAS-Yellow Cameleon 2.1/UAS-Yellow Cameleon 2.1* (in the Ca^{2+} imaging experiment). Embryos were collected within 24 hr of egg laying and heat-shocked at 37°C for 60 min.

Analysis of Courtship Behavior in Mating Chambers

The flies to be tested were reared individually in vials and aged for 4–9 days after eclosion. For courtship assays, a male fly carrying *UAS-dTrpA1* was introduced into a metal-molded chamber (1 cm diameter, 0.3 cm height) and placed on the aluminum block of a thermal cycler. The fly's behavior was then videorecorded while the temperature was increased and decreased. The temperature of mating chamber was maintained at 22°C for 5 min and increased at 1°C every 5 min afterwards. During these temperature shifts, courtship behaviors in the absence of a courtship target (single male assays) were observed. The locomotor speed of the test flies in single male assays was measured automatically with Move-tr/2D Ver.7.2 (Library Co., Tokyo, Japan). The event frequency (Figure 1B) was calculated as the proportion of time the male spent performing each courtship action during the observation period (5 min for each temperature condition). The perievent histograms (Figures 1C and 1D) were constructed by using the midpoint of every tapping event as reference time zero. For a 6 s period staring 3 s before and ending 3 s after the midpoint of every tapping event, fly behavior was analyzed at every 0.1 s time point. This analysis yielded 61 data points associated with a single tapping event that served as the time-zero reference, defining a single data set. The perievent histograms represent the probability distribution of each courtship element with time. If, for example, licking was observed at the 0.1 s period between 2 s and 2.1 s before time zero in 30 out of 90 data sets, the probability of observing licking at this time point is calculated as 30/90 = 0.3. The perievent histogram illustrates the average pattern of temporal organization of courtship elements, allowing quantitative as well as qualitative comparisons of courtship behavior displayed by males of different genotypes or under different conditions. The probability distributions obtained with five different males were used to construct a perievent histogram shown in Figures 1E and 1F with the mean ± standard error of the mean (SEM) values for behaviors induced forcibly by *dTrpA1* in conjunction with *fru*^{NP21} (Figure 1E) and those induced naturally by a female (Figure 1F). The number of observed tapping events was 101.4 ± 9.9 for the male flies with *dTrpA1* expression in *fru*^{NP21}-positive neurons and 39.8 ± 5.4 for the males courting an immobilized female.

Courtship behaviors of male flies that carried MARCM clone neurons with the expression of *dTrpA1* were observed after the temperature increase from 22°C to 35°C. The temperature of mating chamber was maintained at 22°C for 5 min and switched to 35°C for a subsequent 10 min. Based on these observations, the mosaic flies were classified into two groups: courters, who displayed unilateral wing extension and/or tapping, and noncourters, who displayed neither of these activities. Both groups were subjected to immunohistochemistry to determine which neurons were expressing *dTrpA1*.

Courtship behaviors of male flies that carried MARCM clone neurons with the expression of *shi*^{ts} were observed as follows. A mosaic male fly was placed alone in a chamber at 30°C for 10 min and then a virgin female fly was introduced into the chamber, the temperature of which was maintained at 30°C for the following 10 min and switched to 20°C for a subsequent 10 min. The courtship index (CI) was calculated for the last 5 min of each 10 min temperature period. The CI was defined as the proportion of time the male spent performing courtship during the 5 min observation period. We examined, in total, 288 mosaic flies with *shi*^{ts} expression for quantitative comparisons of courtship activities among the flies in which P1 or P2b was inhibited unilaterally or bilaterally. In this study, we obtained 66 flies with MARCM clones in P1 and 66 flies with MARCM clones in P2b. Among these, 14 flies carried MARCM clones in both P1 and P2b. Bilateral clones were observed in four (P1) and five (P2b) brains. Sixty-two flies had unilateral P1 clones while 61 flies had unilateral P2b clones in their brains. One hundred and seventy flies carried neither a P1 nor a P2b MARCM clone.

Fixation and immunohistochemical staining were carried out as described previously (Kimura et al., 2005), with the following antibodies and dilutions: rabbit polyclonal anti-GFP (1:1,000; Molecular Probes, Eugene, OR), mouse monoclonal nc82 (1:20), Alexa546-conjugated goat anti-mouse IgG (1:200; Invitrogen, Carlsbad, CA), and Alexa488-conjugated goat anti-rabbit IgG (1:200; Invitrogen). Stacks of optical sections at 1 or 2 μm were obtained with an LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany) and were processed with ImageJ software (ver. 1.40 g). To evaluate the possibility of synaptic connections between two neuronal populations, the image of a P2b mosaic brain was reformatted on that of a P1 mosaic brain by IGSRegistration Tools as described in Jefferis et al., (2007) and Cachero et al., (2010).

Optical Locomotion Tracking System

Flies were attached to the tip of an insect pin of 0.2–0.4 mm in diameter by using UV glue and were then placed on a Styrofoam ball approximately 5 mm in diameter (Buchner, 1976). The ball was floated stably on a jet of air from the bottom of the apparatus and thus could be easily rotated by the fly's locomotion. The fly's locomotion patterns were measured as the rotation of the ball along the yaw (Δyaw) and pitch axes (Δpitch), which represented the fly's right-left turns and forward-backward movements, respectively. The rotation of the ball was recorded at 100 Hz by detecting changes in the surface texture of the ball along two orthogonal axes (Δx, Δy) using an optical sensor taken from a computer mouse. The sensor was set to point to the posterior end of the ball relative to the fly body, so that Δx and Δy were in proportion to Δyaw and Δpitch, respectively. The behavior of the male fly and the position of the target fly were simultaneously recorded at 100 fps with a high-speed camera (MotionScope M3; Integrated Design Tools, Tallahassee, FL). The acquisitions of the tracking data and the fly images were matched in time by a trigger signal generated by a custom controller device, and the program was written in the python programming language.

Analysis of Courtship Behavior under Tethered Conditions

Canton-S, singly raised wild-type males aged 5–7 days posteclosion were attached to the tip of a metal pin under cold anesthesia. The flies were allowed to grab a Styrofoam ball and were left in a humid chamber for at least 2 hr for recovery. After the recovery, the fly was set on the floating Styrofoam ball on the tracking system for courtship assays. The male was subjected to a single touch on the foreleg with the abdomen of a target fly attached to a micromanipulator arm. For ensuring a single, gentle touch, the contact stimulation was given when the male was at rest on the ball and was not exhibiting grooming,

walking, or running behaviors. Just after the touch, the male was exposed to a visual stimulus composed of seven to ten reciprocating horizontal movements by the target fly for 30 s. As a negative control, only the visual stimulus of the moving virgin female body was applied with no preceding touch on the foreleg. The locomotion patterns and behavior of the fly were recorded before, during, and after the application of the touch and visual stimuli. By analysis of video images, the coordinates of the test male and target fly were obtained frame-by-frame with the object tracking function of the control software for a high-speed camera, MotionProX (Integrated Design Tools), and the time windows during which the moving target fly was right or left of the midline of a subject male were determined. The following activity was evaluated by the following index (FI), which was defined as $FI = (\sum|\Delta x_{ipsi}| - \sum|\Delta x_{contra}|)/(\sum|\Delta x_{ipsi}| + \sum|\Delta x_{contra}|)$, where Δx_{ipsi} and Δx_{contra} represent the Δx signals in the direction of and in the direction against, respectively, the presented target abdomen. The stimulus females and males were collected immediately after eclosion and maintained in a group of approximately ten flies of the same sex in a vial for 5 days. The wings and legs of the stimulus flies were removed under cold anesthesia and glued to an insect pin held by a micromanipulator, then used as stimuli within 5 hr after preparation.

In Vivo Ca^{2+} Imaging

A 5- to 6-mm hole was punched in a plastic coverslip, and the punched area was covered with a polyethylene film. The test fly was fixed to the polyethylene film at its dorsal thorax and neck under cold anesthesia. After the fly was allowed to recover from the surgery in a humid chamber for more than 2 hr, the polyethylene film covering the head was cut together with the head capsule underneath by using an injection needle. For obtaining better visual access to the brain, fat bodies were removed and air sacs were carefully unglued from the brain and set aside with fine forceps. Saline covering the exposed brain was removed and transparent silicone gel (KWIK-SIL; World Precision Instruments, Sarasota, FL) was immediately overlaid to reduce the brain movements and to seal the head opening. The brain was observed on a fluorescent microscope (Axio Imager Z1; Carl Zeiss) equipped with a water immersion lens (40×, NA = 0.8; Carl Zeiss). The fluorescent images were captured with a multichip CCD camera (C7780-20; Hamamatsu Photonics, Hamamatsu, Japan). The light source was a high-pressure mercury lamp and a 440/20 nm excitation filter, 455 nm long-path dichroic filter, and 460 nm long-path emission filter were used. The emission light was further split by beam-splitting prisms embedded in the CCD camera (460–490 nm for CFP signals and 490–570 nm for YFP signals), and the split light was projected onto separate CCD chips. Images were taken at a rate of 5–10 Hz, with an exposure time of 100–200 ms for each frame. The binning of the CCD chips was set to give a spatial resolution of 1.28 × 1.28 μm/pixel. The stimulant fly bodies were prepared as described in tethered male behavioral experiments. A stimulus was applied to the tethered male by gently touching its foreleg with a stimulant (a fly body or a glass rod with or without fly extract) attached to the holder on a manual micromanipulator. In experiments for comparison of the response magnitudes evoked by different stimuli, each fly was sequentially applied with all the stimuli in random order with interstimulus interval of 3 min. The specimen and stimulant under the objective lens were illuminated with infrared light and videotaped at 30 fps with an infrared camera. A videorecorder for monitoring behaviors and another for obtaining Ca^{2+} images were triggered simultaneously by an electrical signal, and the time codes embedded in the video images were used to determine the temporal correlations between the record of behavior and the record of Ca^{2+} activities.

Image Processing

All images were processed on ImageJ using custom macro programs. First, two series of raw images (YFP and CFP channels) were registered to reduce movement artifacts and filtered by a median filter (size: 2 pixels) to reduce noise. The fluorescence intensity was measured at a circular region of interest (ROI) with a diameter of 10 pixels. For background subtraction, a brain region without YC2.1 expression was chosen and its fluorescence intensity was subtracted from that at the ROIs. For yielding the fractional YFP/CFP ratio change ($\Delta R/R_0$), the baseline value (R_0) of an average YFP/CFP ratio estimated from the 5 frames before the stimulus onset was subtracted from the YFP/CFP ratio at each time point; then the difference (ΔR) was divided by R_0. The fractional changes in YFP ($\Delta Y/Y_0$) and CFP ($\Delta C/C_0$) were obtained by the procedure described for $\Delta R/R_0$. When the maximum $\Delta R/R_0$ was attained within 3 s after the stimulus onset, this peak was judged to be an evoked response. The average $\Delta R/R_0$ value estimated from the 3 frames around the peak response was used for quantitative comparisons of responses.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and five movies and can be found with this article online at doi:10.1016/j.neuron.2010.12.017.

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